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CAT-1-Mediated Arginine Uptake and Regulation of Nitric Oxide Synthases for the Survival of Human Breast Cancer Cell Lines

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ABSTRACT

Growth of the human MCF-7 breast cancer cell line is highly dependent on L-arginine. We have reported that L-arginine, released from extracellular substrates by prolactin (PRL)- and 17β-estradiol (E2)-induced carboxypeptidase-D in the cell membrane, promotes nitric oxide (NO) production for MCF-7 cell survival. Arginine uptake is mediated by members of the cationic amino acid transporter (CAT) family and may coincide with induction of nitric oxide synthase (NOS) for the production of NO. The present study investigated the CAT isoforms and PRL/E2 regulation of CAT and NOS in breast cancer cell lines. Using RT-PCR analysis, CAT-1, CAT-2A, and CAT-2B transcripts were detected in MCF-7, T47D, and MDA-MB-231 cells. The CAT-4 transcript was detected in MDA-MB-231 only. CAT-3 was not detected in any of these cells. PRL and E2 did not significantly alter levels of CAT-1 mRNA and protein, nor CAT-2A and CAT-2B mRNAs in MCF-7 and T47D cells. PRL and E2 also had no effect on the overall uptake of L-[2,3,4,5-H^3] arginine into these cells. However, confocal immunofluorescent microscopy showed that PRL and E2 upregulated eNOS and iNOS proteins, which distributed in the cytoplasm and/or nucleus of MCF-7 cells. Knockdown of CAT-1 gene expression using small interfering RNA significantly decreased L-[2,3,4,5-H^3]-arginine uptake, decreased viability and increased apoptosis of MCF-7 and T47D cells. In summary, several CAT isoforms are expressed in breast cancer cells. The CAT-1 isoform plays a role in arginine uptake and, together with PRL/E2-induced NOS, contribute to NO production for the survival of MCF-7 and T47D cells. J. Cell. Biochem. 112: 1084–1092, 2011. © 2011 Wiley-Liss, Inc.

KEY WORDS: CATIONIC AMINO ACID TRANSPORTER; ARGININE UPTAKE; NITRIC OXIDE SYNTHASES; BREAST CANCER

INTRODUCTION

In the cell, L-arginine is metabolized by arginases to L-ornithine for polyamine biosynthesis, and by nitric oxide synthases (NOS) to L-citrulline and nitric oxide (NO) [Nathan and Xie, 1994; Wu and Morris, 1998; Pance, 2006]. NO is a pleiotropic regulator of many physiological processes, and may also have dual pro- and anti-tumor effects [Beckman and Koppenol, 1996; Geller and Billiar, 1998; Thomsen and Miles, 1998; Fukumura et al., 2006]. We have previously reported that L-arginine, either added exogenously or released from extracellular substrates by a prolactin (PRL)- and 17β-estradiol (E2)-induced carboxypeptidase-D (CPD) located in the plasma membrane, promotes intracellular production of NO in the human MCF-7 breast cancer cell line. The elevated levels of NO increase MCF-7 cell viability and inhibit cell apoptosis [Abdelmagid and Too, 2008]. The cellular uptake of L-arginine is mediated by the cationic amino acid transporter (CAT) family of proteins [Mann et al., 2003]. CAT-mediated arginine uptake is often coordinated with the expression and/or activity of the NOS enzymes for the production of NO [Nathan and Xie, 1994; Pance, 2006]. It is not known whether PRL/E2 upregulation of CPD is co-ordinated with CAT and NOS levels to modulate L-arginine uptake and NO production for the survival of breast cancer cells.

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The uptake of L-arginine, L-lysine, and L-ornithine is mediated by several transport systems which include the Na^+-independent systems y^+, y^+L, $b^{0,+}$, b^+, and the Na^+-dependent system $B^{0,+}$ [Closs, 2002; Mann et al., 2003]. System y^+ is the principal cationic amino acid transport system expressed in NO producing cells and thus is believed to play a key role in regulating the intracellular supply of L-arginine for NOS. There are five system y^+ carrier proteins, CAT-1, CAT-2A, CAT-2B, CAT-3, and CAT-4, which are classified as members of the solute carrier family 7 (SLC7). CAT-1, CAT-3, and CAT-4 are encoded by separate genes named *SLC7A1*, *SLC7A3*, and *SLC7A4*, respectively. Transport of L-arginine, L-lysine, and L-ornithine via CAT-1 is pH-independent and is stimulated by substrates on the *trans* side of the cell membrane, in an effect termed *trans*-stimulation, and by membrane hyperpolarization [Closs, 2002; Mann et al., 2003]. With the exception of the liver, CAT-1 is expressed in almost all cell types. CAT-2A and CAT-2B are splice variants of *SLC7A2*, and differ from each other only in a stretch of 42 amino acids. CAT-1, -2A, and -2B are glycosylated, suggesting that they are located in the plasma membrane [Mann et al., 2003]. CAT-2A is predominantly expressed in the liver whereas CAT-2B is usually induced under inflammatory conditions in a variety of cells. CAT-2A is a low-affinity carrier for cationic amino acids, and unlike the high-affinity CAT-1, is relatively insensitive to *trans*-stimulation [Mann et al., 2003]. CAT-3 is brain-specific in rat and mouse [Hosokawa et al., 1997; Ito and Groudine, 1997] whereas human CAT-3 is present in the brain but preferentially expressed in peripheral tissues such as the thymus, where it is most abundant, and in the mammary gland, uterus, and testis [Vekony et al., 2001]. A cDNA, *SLC7A4*, encoding CAT-4, has been identified in human placenta with 41%–42% sequence identity to members of the CAT family [Sperandeo et al., 1998]. However, since the expression of CAT-4 in the plasma membrane is not sufficient to induce amino acid transport activity in *Xenopus laevis* oocytes or human cells, it has been suggested that CAT-4 is either not an amino acid transporter or that it requires additional factors to be functional [Wolf et al., 2002].

System y^+L transports cationic amino acids in a Na^+-independent manner as well as neutral amino acids with high affinity in a Na^+-dependent manner [Deves and Boyd, 1998]. System y^+L may serve as an efflux pathway for cationic amino acids since high activity of the system can lead to L-arginine depletion [Mendes Ribeiro et al., 1999]. However, there is also evidence for the involvement of system y^+L in NO synthesis in human platelets and endothelial cells [Arancibia-Garavilla et al., 2003; Signorello et al., 2003].

There are three NOS isoforms [Nathan and Xie, 1994; Pance, 2006] that have been localized in the cytoplasm, nucleus and subcellular compartments in various cell types [Shaul et al., 1996; Gilchrist et al., 2004; Saini et al., 2006]. Constitutive neuronal (nNOS/NOSI) and endothelial (eNOS/NOSIII) NOS are classified as Ca^{2+}-dependent, and they raise small amounts of NO. However, Ca^{2+}-independent activation of eNOS by E2, isoflavones, or isometric contraction has been reported in human aortic and/or umbilical cord endothelial cells [Caulin-Glaser et al., 1997; Fleming et al., 1999; Joy et al., 2006]. The third isoform, inducible NOS (iNOS/NOSII), is Ca^{2+}-independent and, upon induction by cytokines or bacterial lipopolysaccharides (LPS), raises high levels of NO. Depending on its intracellular concentration, duration of production and/or the microenvironment, NO may either stimulate or suppress growth of a variety of tumor cells, including breast cancer cells [Bani et al., 1995; Mortensen et al., 1999; Reveneau et al., 1999].

The present study investigated the y^+/CAT isoforms expressed in human breast cancer cell lines, MCF-7, T47D, and MDA-MB-231. We determined the effects of PRL and E2 on CAT expression and arginine uptake, and the role of CAT-1 for the survival of MCF-7 and T47D cells. Lastly, we determined PRL and E2 regulation of NOS expression and subcellular distribution in MCF-7 cells.

MATERIALS AND METHODS

ANTIBODIES

The primary antibodies used were polyclonal rabbit anti-CAT-1 (Abcam Inc., Cambridge, MA), anti-eNOS, and anti-iNOS (Santa Cruz Biotechnology, Santa Cruz, CA), and anti-actin (Sigma–Aldrich Canada Ltd., Oakville, Ontario, Canada). Secondary donkey anti-rabbit IgG-horse radish peroxidase (HRP) conjugate was from Amersham Pharmacia Biotechnology (Baie d'Urfe, Quebec, Canada). AlexaFluor488 goat anti-rabbit conjugate was from Molecular Probes, Inc. (Eugene, OR).

CELL CULTURES

Human MCF-7, T47D, and MDA-MB-231 breast cancer cell lines were cultured in a humidified, 5% CO_2 atmosphere at 37°C. MCF-7 cells were maintained in high glucose (25 mM) Dulbecco's modified Eagle's Medium (DMEM) containing 10% v/v heat-inactivated fetal bovine serum (FBS) and supplemented with 1xMEM non-essential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. T47D and MDA-MB-231 cells were maintained in high glucose DMEM containing 10% v/v heat-inactivated FBS, 5 mM HEPES, 2 mM L-glutamine, and penicillin/streptomycin. For some experiments, actively growing cells, about 50–60% confluent, were washed twice with phosphate-buffered saline (PBS) and made quiescent for 24 h in phenol red-free DMEM containing 1% horse serum (HS; lactogen/PRL-free) prior to PRL treatment or 1% charcoal stripped-FBS (steroid-free) prior to E2 treatment. Suspension cultures of human Burkitt lymphoma-derived Daudi B cell line were maintained in RPMI-1640 containing 10% FBS. All culture reagents were from Invitrogen Canada Inc. (Burlington, Ontario, Canada).

REVERSE TRANSCRIPTION AND POLYMERASE-CHAIN REACTION (RT-PCR)

Total RNA was isolated using GenElute Mammalian Total RNA miniprep kit (Sigma–Aldrich). Reverse transcription of total RNA (1 μg) and amplification by PCR were performed as previously described [Dodd et al., 2000]. All PCR reactions were performed within the linear range of amplification. Primer pairs for four of the human CAT genes have been reported previously [Dye et al., 2004] and were: CAT-1, 5′-ATC-TGC-TTC-ATC-GCC-TAC-TT-3′ and 5′-TCT-CTG-CCT-CTG-GTA-AAA-AC-3′ (535 bp product); CAT-2A, 5′-TTC-TCG-TCC-TTC-TGT-TTG-TG-3′ and 5′-TTT-GGG-CTG-GTC-

GTA-AGA-TA-3′ (734 bp product); CAT-2B, 5′-TTT-TCC-CAA-TGC-CTC-GTG-TA-3′, and 5′-CAT-TTG-GGC-TGG-TCG-TAA-GA-3′ (265 bp product); CAT-4, 5′-ATG-GTG-GGC-TCG-GGT-CTC-TA-3′ and 5′-TGC-GGA-TGC-TGT-GGC-TGA-AC-3′ (304 bp product). Primers for human CAT-3 were: 5′-GGC-CTC-CTG-TTC-CGT-GTA-CTT-3′ and 5′-CCT-GCA-ACT-CCA-CTT-CTT-CC-3′ (238 bp product). Human β-actin: 5′-AAA-CTG-GAA-CGG-TGA-AGG-TG-3′ and 5′-AGA-GAA-GTG-GGG-TGG-CTT-TT-3′ (171 bp product), was used as a control. RT-PCR products were resolved in 1–2% agarose gels.

TRANSFECTIONS OF SMALL INTERFERING RNA (siRNA)

Non-targeting siRNA (siNT) and siCAT-1 (Invitrogen), at a concentration of 10 nM, were transfected into MCF-7 or T47D cells using RNAiMAX reagent (Invitrogen) following the manufacturer's instructions. After 24 h, cells were seeded onto chambered slides or 24-well plates, and allowed to grow for another 24–48 h prior to RNA isolation or the start of experiments. Knockdown of gene expression was confirmed by RT-PCR.

CATIONIC AMINO ACID TRANSPORT ASSAY

Transport assay for L-arginine was performed as previously described for a variety of cell lines [Durante et al., 1996; Simmons et al., 1996; Racke et al., 1998; Wu et al., 2007], including breast cancer cells [Wu et al., 2007], with modifications. Briefly, cells were seeded onto 24 well plates (40,000 cells/well). Following treatment with hormones or siRNA, the cells were washed twice with PBS and incubated in pre-warmed Kreb's buffer (25 mM HEPES, pH 7.4, 119 mM NaCl, 4.6 mM KCl, 11 mM glucose, 1.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 15 mM $NaHCO_3$, 1.2 mM NaH_2PO_4,) for 30 min at 37°C. Transport assays were initiated by incubating cells in Kreb's buffer containing L-[2,3,4,5-H^3] arginine monohydrochloride (50 μM; 1–2 μCi/ml) for 1 min, which was within the linear portion of the uptake curve (data not shown). In some experiments, L-lysine was added prior to the addition of L-[2,3,4,5-H^3] arginine to inhibit L-arginine transport. Transport activity was terminated by aspirating the medium and rapidly washing the cells with ice cold PBS containing 10 mM L-arginine (stop solution), followed by extraction of the monolayer with 0.2 ml ethanol for 30 min at 37°C. Cell extracts were centrifuged at 14,000 rpm for 4 min and a 100-μl aliquot was added to 10 ml of Ready Safe liquid scintillation cocktail for counting (Beckman Coulter, Fullerton, CA). To correct for non-specific uptake or binding to the cell surface, cells in identical wells were incubated in uptake buffer containing 20 mM L-arginine, the fraction of the radioactivity associated with the cells was determined, and this fraction was subtracted from each data point.

MEASUREMENT OF CELL VIABILITY: MTS ASSAY

Cells were seeded into 24-well plates (50,000 cells/well). After 1–6 days, the MTS assay (CellTiter 96® AQueous Non-Radioactive Proliferation Assay; Fisher Scientific, Ltd., Nepean, ON, Canada) was performed following the manufacturer's instructions.

MEASUREMENT OF APOPTOSIS: ANNEXIN-V STAINING

Cells were seeded onto chambered slides. After 24 h, annexin-V staining was performed following the manufacturer's instructions (Roche Applied Sciences, Laval, QC, Canada). Fluorescent staining was examined using a Zeiss Axiovert 200 fluorescent microscope and images were captured using an AxioCam HRc camera. Stained cells were counted in several fields of 300–900 cells/treatment.

WESTERN ANALYSIS

Cells were homogenized in RIPA buffer containing protease inhibitors and sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE; 10% gels) was performed as previously described [Dodd et al., 2000]. For CAT-1, 40–50 μg protein/lane was used for SDS-PAGE. For NOS, 100 μg protein/lane was used to compensate for very low levels of the eNOS transcript in MCF-7 cells (data not shown). Immunoblotting was performed using anti-CAT-1 (1:1000), anti-eNOS (1:100), anti-iNOS (1:100) antibodies, and donkey anti-rabbit IgG-HRP conjugate (1:2,500). Actin was used as a control. Immunoreactive signals were detected using Immobilon Western HRP Substrate Kit (MILLIPORE, Billerica, MA).

CONFOCAL IMMUNOFLUORESCENT MICROSCOPY

Chambered slides (VWR) were coated with poly L-lysine prior to seeding of MCF-7 cells (50,000 cells /well). The cells were fixed and stained as described previously [O'Malley et al., 2005]. Immunostaining was performed using anti-eNOS (1:40) or anti-iNOS (1:30) antibodies, and AlexaFluor 488 goat anti-rabbit conjugate (1:50). Confocal z-stack images were acquired using a Zeiss LSM 510

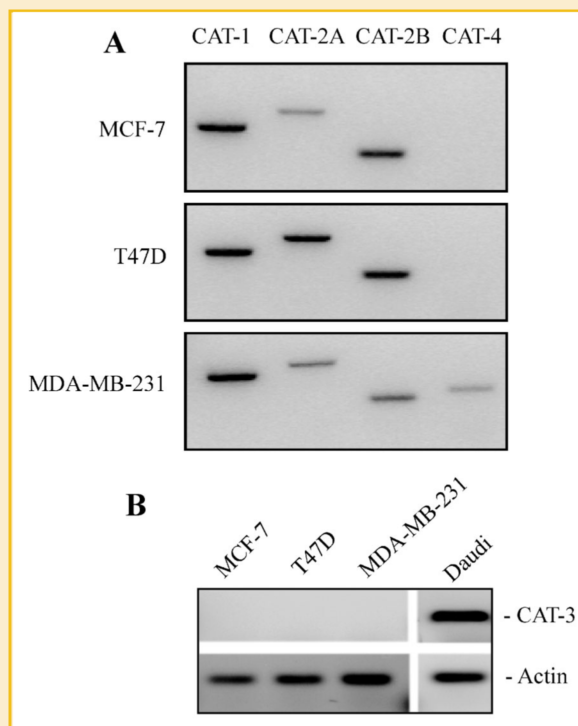

Fig. 1. CAT isoforms in breast cancer cells. Actively growing MCF-7, T47D, and MDA-MB-231 cells were cultured in their respective complete medium. Total RNA was isolated for RT-PCR analysis using specific primers as described in Materials and Methods. (A) Detection of CAT-1, CAT-2A, CAT- 2B, and/or CAT-4 transcripts. (B) Detection of CAT-3. Daudi B-lymphoma cells were a positive control. Actin was used as a loading control.

microscope. Fluorescent images were converted to grayscale and inverted, using Adobe Photoshop. NIH ImageJ was used to quantify the relative area of fluorescence in each image. A threshold, chosen to best encompass fluorescent staining but exclude background fluorescence, was applied to all the images. For each image, the area above the threshold was measured and plotted.

STATISTICAL ANALYSIS

Statistical analyses were performed using Graph Pad Prism. Results were expressed as mean ± SEM. Analysis of variance and Fisher's protected least significant difference test were used to compare the means. *P*-values of <0.05 was considered significant.

RESULTS

CAT ISOFORMS IN BREAST CANCER CELL LINES

RT-PCR analysis detected CAT-1, CAT-2A, and CAT-2B transcripts in MCF-7 and T47D cells. MDA-MB-231 cells expressed CAT-1, CAT-2A, CAT-2B, and CAT-4 (Fig. 1A). The CAT-3 transcript was not detected in any of the three breast cancer cell lines but was present in Daudi B-lymphoma cells, which served as a positive control (Fig. 1B). Daudi cells have previously been shown to express CAT-3 in a human multiple tissue expression array [Vekony et al., 2001].

PRL AND E2 HAVE NO SIGNIFICANT EFFECT ON CAT mRNA/PROTEIN LEVELS

We have reported that PRL and E2 stimulate the expression of CPD to release C-terminal arginine from extracellular substrates in MCF-7 cells that were cultured in DMEM containing 25 mM glucose [Abdelmagid and Too, 2008]. The present study investigated whether these hormones also regulate CAT levels under similar culture conditions. MCF-7 and T47D cells express receptors for PRL (PRLR) and E2 (ER) whereas MDA-MB-231 cells are ER-negative [Weigel and deConinck, 1993] and have lower levels of the PRLR than the other two cell lines [Shiu, 1979]. Therefore, MCF-7 and

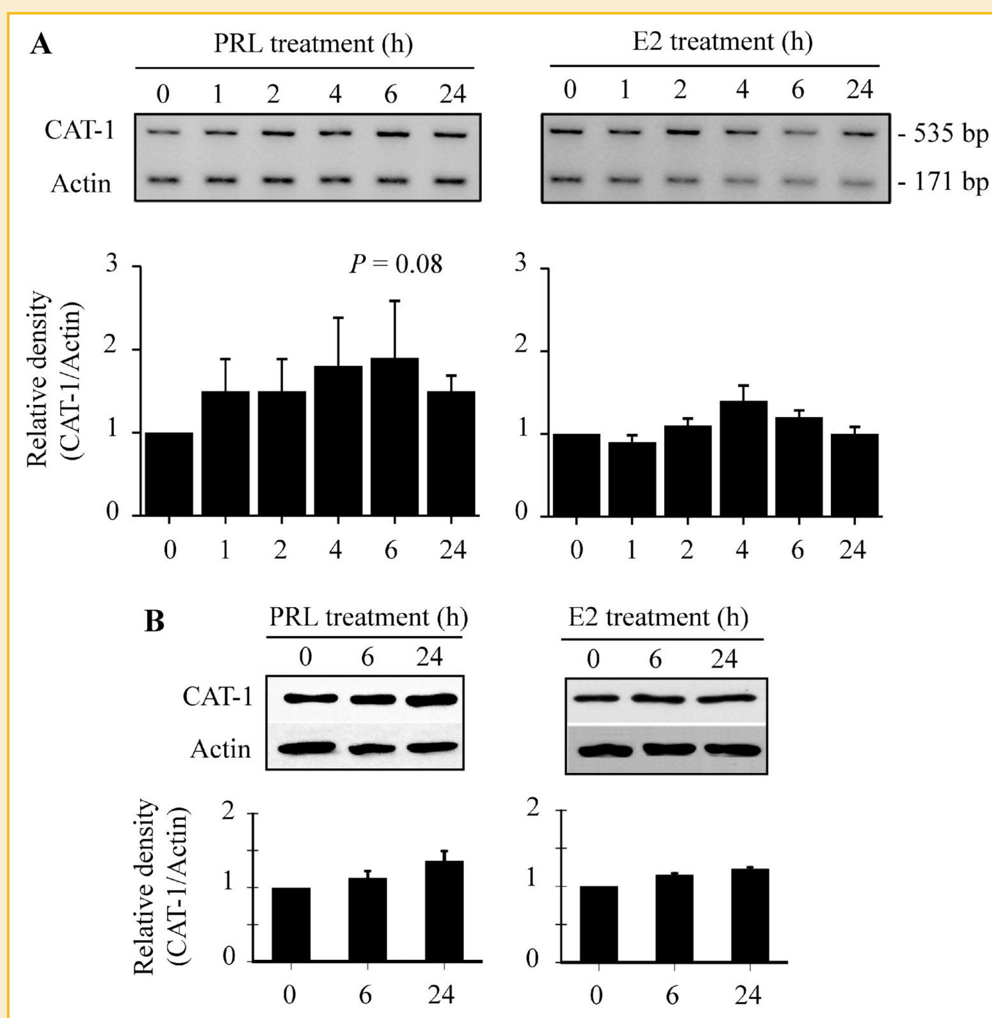

Fig. 2. PRL and E2 have no significant effect on CAT-1 mRNA/protein levels. Quiescent MCF-7 cells were treated with PRL or E2. At the indicated times, (A) total RNA was isolated for RT-PCR analysis and (B) total cell lysates prepared for Western analysis. In (A) and (B) lower panels, densitometric analyses were performed for the CAT-1/actin ratio. Mean ± SEM (n = 3). $P = 0.08$ at 6 h indicated insignificant change in CAT-1 mRNA levels. Protein levels were also unaltered.

T47D cells were further studied, and after they were made quiescent the cells were treated with PRL or E2 for specific times. Semi-quantitative RT-PCR analysis showed no significant change in CAT-1 mRNA levels following hormonal treatment (Fig. 2A). Western analysis also showed no change in CAT-1 protein levels (Fig. 2B). PRL and E2 treatment also had no effect on CAT-1 mRNA/protein levels in T47D cells (data not shown). Neither hormone had any effect on CAT-2A and CAT-2B mRNA levels in the two cells lines (data not shown).

PRL AND E2 HAVE NO EFFECT ON ARGININE UPTAKE

The CAT proteins mediate cellular uptake of L-arginine. The hormonal effect on L-arginine uptake was next investigated. MCF-7 and T47D cells were treated with PRL or E2, and then pulsed with L-[2,3,4,5-H^3] arginine. PRL and E2 had no effect on L-arginine uptake into these cells, as compared to untreated controls, which showed an uptake of about 0.6–1.2 nmol L-arginine/mg protein/min (Fig. 3). Other studies have reported that the Na^+-dependent transport of L-arginine into MCF-7 cells occurred at about the same rate (i.e., ~0.7 nmol/mg protein/min) [Wu et al., 2007].

siCAT-1 DECREASES ARGININE UPTAKE

Since CAT-1 is believed to conform best to system y^+ and is ubiquitously expressed [Closs, 2002; Mann et al., 2003], the role of CAT-1 in arginine uptake was further analyzed by knocking down CAT-1 gene expression using siRNAs. Western analysis showed that siCAT-1 decreased CAT-1 protein levels by about 45%–50% in MCF-7 and T47D cells, as compared to non-targeting siNT (Fig. 4A). RT-PCR analysis showed the specificity of siCAT-1 which decreased CAT-1 gene expression only, by about 40%–50%, but had no effect on the mRNA levels of CAT-2A, CAT-2B, or actin in similarly transfected MCF-7 cells (Fig. 4B). Knockdown of CAT-1 gene expression was accompanied by a corresponding decrease in arginine uptake, by about 35% in MCF-7 (Fig. 4C) and 40% in T47D cells (Fig. 4D), as compared to cells transfected with siNT. In the presence of L-lysine, arginine uptake was decreased by another 40%–45% in both cell lines (Fig. 4C,D). Since L-lysine inhibits total arginine uptake, the results suggest the presence of additional cationic amino acid transport systems (e.g., ^+L, $b^{0,+}$, b^+, $B^{0,+}$) in these cells.

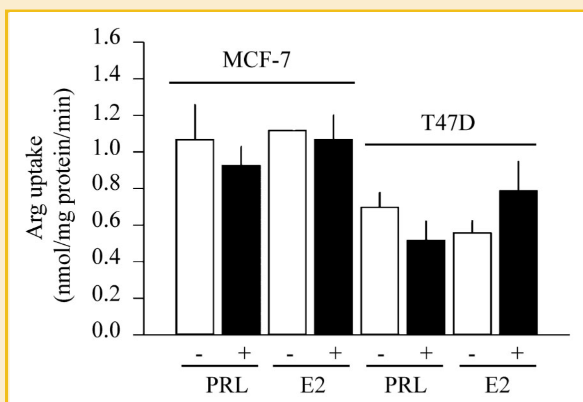

Fig. 3. PRL and E2 have no effect on arginine uptake. Quiescent MCF-7 and T47D cells were treated with PRL or E2 for 6 h. Specific uptake of L-[2,3,4,5-H3] arginine was measured as described in section Materials and Methods, and normalized to nmol/mg protein/min. Mean ± range of two separate experiments, each performed in triplicate. Earlier time points also showed no effect (data not shown).

siCAT-1 DECREASES CELL VIABILITY AND INCREASES CELL APOPTOSIS

The consequences of reduced arginine uptake on cell survival were investigated. MCF-7 and T47D cells were transfected with siCAT-1 or siNT, and then cultured in complete medium for up to 6 days for the MTS assay. In MCF-7 cells, siCAT-1 decreased cell viability by 25% and 50% on day 4 and 6, respectively, as compared to the siNT-transfected cells (Fig. 5A). This was accompanied by an increase in cell apoptosis, as determined using annexin-V staining (Fig. 5B). On day 4, about 55% of siCAT-1-transfected MCF-7 cells were apoptotic, as compared to 17% in untransfected controls or siNT-transfected cells (Fig. 5C). Similar effects were seen in siCAT-1 transfected T47D cells. By 4 day, the knockdown of CAT-1 gene expression decreased cell viability (Fig. 5D) and increased cell apoptosis (Fig. 5E,F), both by about 20%–25%.

PRL AND E2 INCREASE iNOS AND eNOS PROTEIN LEVELS IN MCF-7 CELLS

We next investigated the effects of PRL and E2 on NOS protein levels in MCF-7 cells. Since the NOS enzymes may be found in different compartments within the cell [Shaul et al., 1996; Gilchrist et al., 2004; Saini et al., 2006], confocal microscopy was used to visualize hormonal effects on the levels and subcellular distribution of NOS. Western analysis of MCF-7 cell lysates showed a single immunoreactive band for eNOS and iNOS, respectively (Fig. 6A). Confocal microscopy of actively growing cells also showed that the immunofluoresence produced by these antibodies was decreased by blocking peptides (Fig. 6B), thus indicating antibody specificity.

Quiescent MCF-7 cells, growth-arrested either in 1% HS (PRL-free) or 1% charcoal-stripped FBS (E2-free) for 24 h, were treated with PRL or E2, respectively. PRL treatment increased eNOS-specific immunofluorescence in the cytoplasm and cell nucleus at 3 h, but redistributed eNOS to the cytoplasm at 6 and 24 h (Fig. 6C, *upper panel*). PRL treatment also increased levels of iNOS, which had a punctuate distribution throughout the cell at 3–24 h (Fig. 6C *lower panel*). PRL-stimulated eNOS- and iNOS-specific immunofluorescence was quantified (Fig. 6D). In comparison, E2 treatment increased eNOS-specific immunofluorescence in the cytoplasm and nucleus at 3–24 h (Fig. 6E, *upper panel*) and increased iNOS at 3 h (Fig. 6E, *lower panel*). E2-stimulated iNOS- and eNOS-specific immunofluorescence was also quantified (Fig. 6F). The differences in immunofluoresence between control cells at time 0 (Fig. 6C, E) was probably due to the two different arrest medium that were used.

DISCUSSION

The present study detected mRNAs for CAT-1, CAT-2A, and CAT-2B in MCF-7 and T47D cells, as well as CAT-4 in MDA-MB-231 cells. To our knowledge, this is the first report of the CAT isoforms in human

breast cancer cell lines. CAT-3 has been reported in normal human mammary tissues [Vekony et al., 2001] but we did not detect the CAT-3 transcript in any of the three breast cancer cell lines. PRL and E2 treatment had no significant effect on CAT-1 mRNA and protein levels, nor on the levels of CAT-2A and CAT-2B. However, when CAT-1 gene expression was knocked down using siRNAs, L-arginine uptake was significantly decreased in both MCF-7 and T47D cells, and this was accompanied by a decrease in cell viability and an increase in apoptosis. Therefore, our study shows that CAT-1 plays a role in L-arginine uptake, which contributes to the survival of breast cancer cells.

Gene expression of the CAT isoforms may be affected by glucose concentrations. In our studies, MCF-7 cells were maintained in 10% FBS in high D-glucose (25 mM) DMEM. Other researchers have reported that 25 mM D-glucose induces maximal L-arginine transport, which may or may not be accompanied by a detectable change in CAT-1 and CAT-2B mRNA levels in human umbilical vein endothelial cells [Mann et al., 2003]. However, later studies showed that high concentrations of D-glucose increases L-arginine transport, CAT-1 mRNA expression and eNOS activity in these cells [Sobrevia and Gonzalez, 2009]. On the other hand, *CAT-1* gene expression can be induced by glucose deprivation, which causes a dramatic increase in CAT-1 mRNA and protein levels, and stimulation of L-arginine uptake in human C6 glioma cells [Fernandez et al., 2002]. Under our culture conditions, PRL and E2 treatment upregulate CPD mRNA and protein levels [Abdelmagid and Too, 2008], iNOS and eNOS protein levels (Fig. 6) in MCF-7 cells, but without any significant effect on the levels of CAT-1 (Fig. 2) nor CAT-2A/-2B (data not shown) in MCF-7 and T47D cells.

MCF-7 cell growth has previously been reported to be highly dependent on L-arginine, and this has been attributed to intracellular arginase [Caso et al., 2004]. Arginase catalyzes the

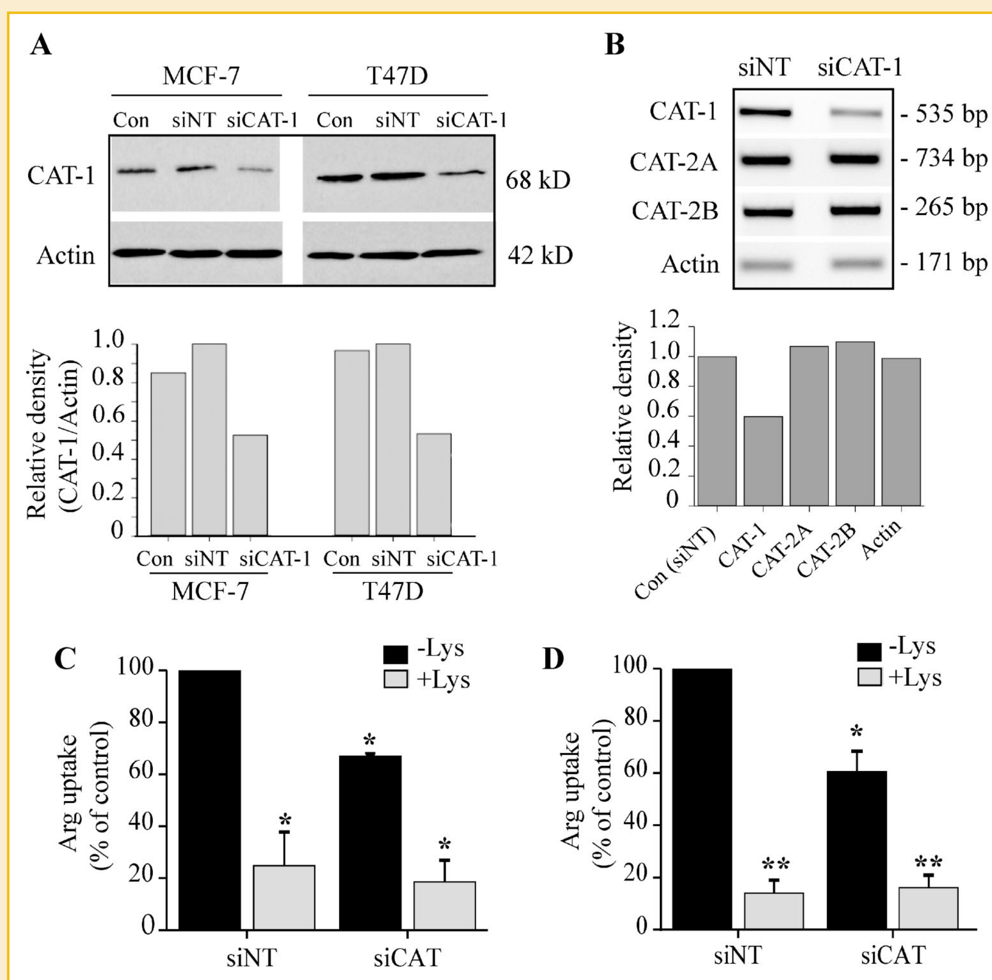

Fig. 4. siCAT-1 decreases arginine uptake. MCF-7 and T47D cells were transfected with siCAT-1 or non-targeting siNT, and cultured in complete medium for 48 and 72 h before RNA extraction or 72 h prior to measuring arginine uptake. (A) Knockdown of CAT-1 gene expression at 48 h was confirmed using Western analysis (upper panel) and the CAT-1/actin ratio was determined by densitometry (lower panel). The CAT-1 protein was decreased to the same extent at 72 h (data not shown). (B) RT-PCR analysis showed that siCAT-1 decreased mRNA levels of CAT-1 but not that of CAT-2A, CAT-2B, or actin (upper panel). Densitometric analysis was performed for each protein, with the protein level of its corresponding control (cells that received siNT) given a unit of one (lower panel). (C,D) Arginine uptake was measured in (C) MCF-7 and (D) T47D cells, ±L-lysine (Lys). Lys (20 mM) was added to inhibit total arginine uptake. Cells transfected with siNT/-Lys was set as 100%. Mean ± SEM of three separate experiments, each done in duplicate. $^{**}P < 0.0001$; $^{*}P < 0.05$, showed significant decrease compared to siNT/-Lys cells.

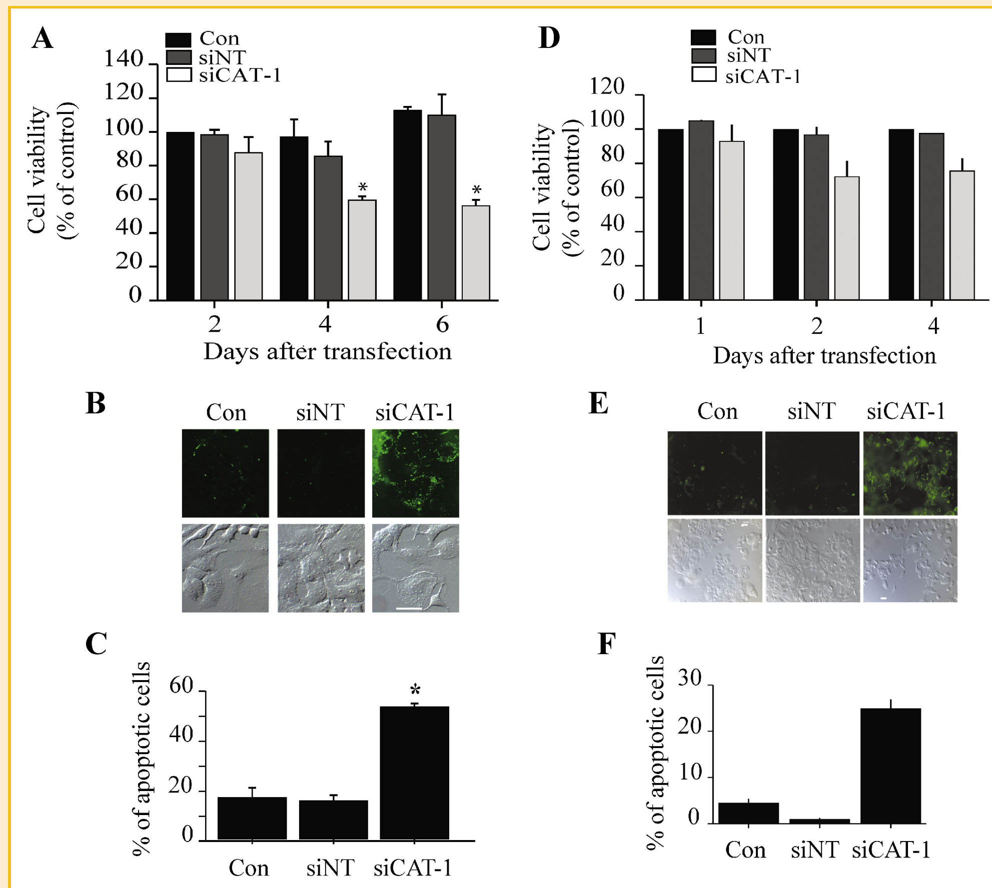

Fig. 5. siCAT-1 decreases cell viability and promotes apoptosis. MCF-7 (A,B,C) and T47D (D,E,F) cells were transfected with siNT or siCAT-1 as in Fig. 4. (A,D) MTS assays were performed on the indicated days after transfection. The viability of untransfected cells (Con) was set as 100% (day 2 for MCF-7 and day 1 for T47D). Compared to siNT-transfected cells, cells transfected with siCAT-1 were less viable. (A) Mean ± SEM (n = 3); $^{*}P < 0.0001$ showed significant decrease compared to Con. (D) Mean ± range of two experiments, each in triplicate. (B,E) Annexin-V staining, performed after 4 days, showed that siCAT-1 increased cell apoptosis. Upper panel: annexin-V staining; lower panel: DIC microscopy, bar = 20 μm. (C,F) The percent of apoptotic cells was plotted. (C) Mean ± SEM (n = 3); $^{*}P < 0.0001$ as compared to Con or siNT-tranfected cells. (F) Mean ± range of two experiments, each in triplicate. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

irreversible conversion of arginine into ornithine, and the progressive depletion of arginine from the culture medium could potentially inhibit protein synthesis and cell growth [Caso et al., 2004]. Alternately, the high arginine requirement of MCF-7 cells could be that L-arginine is the substrate of the enzyme NOS for the production of NO. We have shown that increased NO production promotes MCF-7 cell survival [Abdelmagid and Too, 2008]. In fact, MCF-7 cells have strong Ca^{2+}-dependent NOS activity but very low arginase activity [Singh et al., 2000]. Since eNOS, not iNOS, is Ca^{2+}-dependent [Wu and Morris, 1998], the conversion of L-arginine to NO by eNOS may contribute significantly to the growth of MCF-7 cells.

Our present study showed that PRL and E2 treatment elevated both eNOS and iNOS protein levels in MCF-7 cells. Using confocal microscopy, we have demonstrated not only hormonal-stimulation of NOS-specific immunofluorescence but subcellular redistribution as well. The PRL-induced redistribution of eNOS from the nucleus and cytoplasm at 3 h to the cytoplasm at 6–24 h is intriguing, but the mechanism for this is not known. Other studies have also localized NOS in the cytosol and nucleus, and the intracellular trafficking of NOS has been attributed to post-translational modifications, such as myristylation, palmitoylation [Shaul et al., 1996], and tyrosine phosphorylation [Pan et al., 1996], and to the interactions of the NOS isoforms with regulatory proteins [Kone et al., 2003].

The coordinated stimulation of CAT-mediated arginine uptake with either NOS expression or NOS activity has been demonstrated in a number of studies. For example, LPS and interferon-γ (IFN-γ) stimulate the coordinated expression of CAT-2B and iNOS in rat astrocytes [Stevens et al., 1996]. LPS and IFN-γ also significantly enhance the expression of CAT-1, CAT-2A, CAT-2B, and co-induce iNOS in rat aortic smooth muscle cells [Baydoun et al., 1999]. The cytokine-dependent production of NO by rat cardiomyocytes is a consequence of increased expression of iNOS and is dependent on the coinduction of CAT-1, CAT-2A, and CAT-2B [Simmons et al., 1996].

We have reported that PRL and E2 upregulates CPD within 2 h of hormonal treatment, leading to increased production of NO in MCF-7 cells [Abdelmagid and Too, 2008]. Although PRL and E2 have no effect on CAT-1 gene expression (Fig. 2), both hormones elevate eNOS and iNOS protein levels within 3 h (Fig. 6). Therefore,

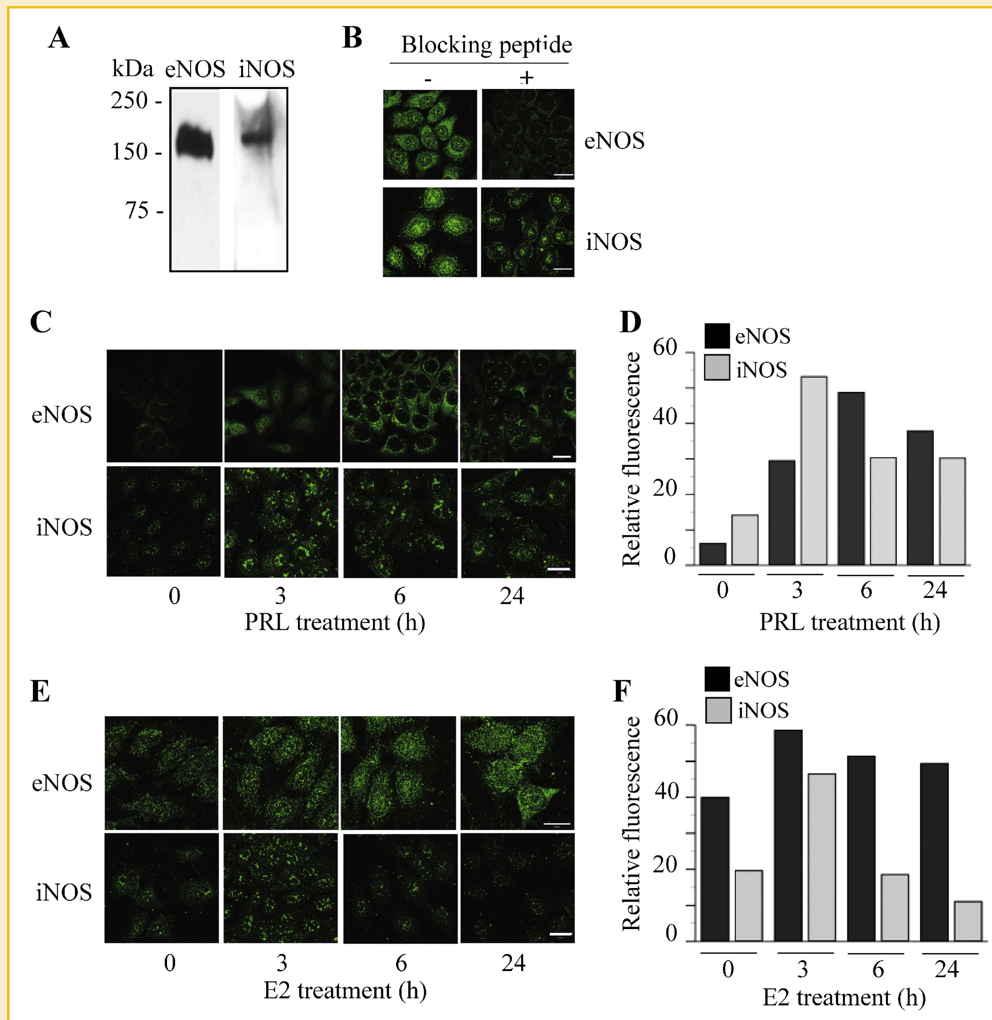

Fig. 6. Confocal immunofluorescent microscopy: PRL or E2 increases NOS protein levels in MCF-7 cells. (A) Western analysis of actively growing MCF-7 cells. (B) Confocal microscopy of actively growing MCF-7 cells incubated with eNOS- or iNOS-specific antibodies, with (+) or without (−) the respective blocking peptide. (C,E) Quiescent MCF-7 cells were treated with (C) 100 ng/ml PRL or (E) 10 nM E2 for the indicated times, followed by confocal microscopy. Bar = 20 μm. Representative of three experiments. (D,F) Immunofluorescence was quantified in (D) PRL- and (F) E2-treated cells as described in section Materials and Methods.

even though arginine uptake in PRL/E2-treated cells is not different from the untreated controls (Fig. 3), the coordinated stimulatory effect of PRL and E2 on CPD and NOS levels would potentially increase the availability of extracellular L-arginine and increase intracellular conversion of L-arginine to NO. Taken together, our studies suggest that PRL/E2-mediated elevation of NO, via CPD and NOS, promotes the survival of breast cancer cells.

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